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ANIMAL AND PLANT HEALTH INSPECTION SERVICE
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SAM - 111

9 CFR 113.145	August 19, 1983	Infectious Bovine Rhinotracheitis Virus
Standard Requirement	New	Agent

SUPPLEMENTAL ASSAY METHOD

FOR

TITRATION OF INFECTIOUS BOVINE RHINOTRACHEITIS VIRUS NEUTRALIZING ANTIBODY

(Constant Virus - Varying Serum Method)

A. SUMMARY

This is an in vitro assay method which employs a cell culture system for determining the antibody titer of serum against Infectious Bovine Rhinotracheitis (IBR) virus.

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B. MATERIALS

1. Cell Cultures

Multiple 6-well disposable plastic plates (35 mm well diameter, Linbro Multi-Dish Disposo-Trays, no endorsement expressed or implied) are seeded with bovine embryonic kidney (BEK) cells (2nd through 5th passage), free of extraneous agents, at a cell count that will produce a monolayer after 3 days of incubation,

2. Growth Medium

The cells are grown in Minimum Essential Medium (MEM) with additives (Appendix 1) at a temperature of 35 to 37 C in an incubator containing an atmosphere of 5% carbon dioxide (CO_2) and a relative humidity of 70 to 80%. Growth medium is not changed unless excess acidity occurs or cells are not growing well.

3. Indicator Virus

National Veterinary Services Laboratories (NVSL) reference IBR virus is used as the indicator virus.

4. Diluent

Maintenance medium (Appendix 2), without serum, is used to make dilutions.

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C. METHODS

1. Dilution of Test Serum

The serum is heat-treated at 56 C for 30 minutes. Serial 2-fold dilutions are made in sterile tubes. Transfers are made with a 1 ml pipette and mixing is done with a mechanical mixer (Vortex or similar type, no endorsement expressed or implied).

a. 0.5 ml diluent is added to tubes 2, 3, 4, and 5.

b. 0.5 ml serum is added to tubes 1 and 2. The pipette is discarded and tube 2 is mixed. Tube 1 contains 0.5 ml of the undiluted serum, and tube 2 contains a 1:2 dilution of serum.

c. 0.5 ml from tube 2 is transferred to tube 3. The pipette is discarded and tube 3 is mixed. Serum in tube 3 is a 1:4 dilution.

d. This process is continued until the desired number of serum dilutions are made. Then, 0.5 ml is discarded from the last dilution tube.

2. Dilution of Indicator Virus

A vial of National Veterinary Services Laboratories (NVSL) reference IBR virus is thawed, mixed, and diluted so that it contains 30 to 70 plaque forming units (PFU) per 0.1 ml.

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3. Serum Neutralization of Virus and Virus Control

a. Five-tenths (0.5) ml of indicator virus is added to each serum dilution tube, mixed, and held at room temperature for 45 minutes to allow for neutralization of virus. The mixing of equal volumes of serum dilution and virus results in a further 2-fold dilution of serum. Thus, the undiluted serum now is a 1:2 dilution, and the initial 1:2 is a 1:4, etc.

b. A virus control is prepared by mixing 1.0 ml of the indicator virus with 1.0 ml of diluent and handled in the same way as the virus-serum mixtures.

4. Inoculation of Cells and Virus Adsorption

a. Before inoculation of the BEK cell monolayers, the growth medium is removed by aspiration with a sterile Pasteur pipette attached to a vacuum tube.

b. Two wells are inoculated with 0.1 ml of a virus-serum mixture.

c. Four wells are inoculated with 0.1 ml of the virus-diluent mixture.

d. In each series of tests, two or more wells containing cell monolayers are maintained as uninoculated controls.

e. Inoculated cells are placed in the CO_2 incubator for one hour to allow for virus adsorption.

5. Overlay and Incubation

When the cells are ready for the overlay, 3 ml of the overlay medium (Appendix 3), at room temperature, is added to each well and the plates are returned to the CO_2 incubator. The plates remain in the incubator undisturbed for 3 to 4 days.

6. Plaque Counting

The cell cultures are prepared and counted as follows:

a. Overlay medium is poured off.

b. One or 2 ml crystal violet solution (Appendix 4) is added to each well and allowed to spread evenly over the monolayers. The plates are allowed to stand at room temperature for a minimum of 10 minutes.

c. The crystal violet solution is washed from the cell monolayers by dipping each plate several times in a container of running cold tap water.

d. Plaques are counted and recorded. The plaques are visible as circular areas in the monolayer where cells have been destroyed by the virus and fail to retain the dye as the uninfected cells do.

D. INTERPRETATION

The plaque reduction titer is the highest serum dilution which causes a 50% or greater reduction in the virus plaque count as compared to the average plaque count of the virus-diluent mixture. Example: Stock virus plaque count is 50. The serum-virus dilution with half (25) the plaques is the titer of the serum.

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APPENDIX

1. Growth Medium:

Lactalbumin hydrolysate or Edamin	0.5 %
L-Glutamine	1.0 %
MEM (Eagle with Earles' salts) q.s. ad	100.0 %
Penicillin	100.0 units/ml
Gentamicin sulfate	50.0 mcg/ml
Streptomycin	100.0 mcg/ml
Amphotericin B	2.5 mcg/ml
Fetal calf serum	10.0 %

2. Maintenance Medium

Lactalbumin hydrolysate or Edamin	0.5 %
L-Glutamine	1.0 %
MEM (Eagle with Earles' salts) q.s. ad	100.0 %
Gentamicin sulfate	50.0 mcg/ml
Amphotericin B	2.5 mcg/ml
Penicillin	100.0 units/ml
Streptomycin	100.0 mcg/ml

3. Overlay (Gum Tragacanth, V.S.P., Fisher Scientific, no endorsement expressed or implied)

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a. Preparation of 2 X gum tragacanth:

(1) The desired volume of deionized water is heated to nearly boiling.

(2) Tragacanth is added (2.0 gm/100 ml for 2%) a little at a time and mixed vigorously with a blender.

(3) A vacuum source is attached to the container to obtain a sustained vacuum overnight. NOTE: The mixture is frothy and will boil out of the flask during autoclaving if air is not removed.

(4) This is sterilized by autoclaving at 15 lbs/psi for 30 minutes. It is then stored at 4 C.

The viscosity of tragacanth does not seem to vary significantly with temperature change. The 2 X tragacanth can be made up in large volumes and stored at 4 C until used.

b. Use of 2 X gum tragacanth:

The needed volume of 2 X tragacanth is added to an equal volume of 2 X medium. This mixture is warmed to room temperature before adding to cell cultures (see Appendix 3. c. for details).

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c. 2% Tragacanth 500.0 ml

10 X MEM (Eagle with Earles' salts, without sodium bicarbonate 100.0 ml

Distilled water 260.0 ml

Serum (goat) 50.0 ml (5%)

7.5% $NaHCO_3$ 30.0 ml

L-Glutamine 10.0 ml

Gentamicin sulfate 50.0 mcg/ml*

Amphotericin B 2.5 mcg/ml**

Penicillin 100.0 units/ml

Streptomycin 100.0 mcg/ml

* 1 ml standard solution (diluted so 1 ml contains 50,000 mcg)

** 1 ml standard solution (diluted so 1 ml contains 2,500 mcg)

4. Preparation of Crystal Violet Stain:

a. Ingredients:

Crystal violet 7.5 gm

Ethyl alcohol (70%) 50.0 ml

Formalin 250.0 ml

Distilled water q.s. to 1000.0 ml

b. Crystal violet is dissolved in ethyl alcohol, then formalin and water are added in that order.